Adverse prognostic effect of homozygous *TET2* mutation on the relapse risk of acute myeloid leukemia in patients of normal karyotype

Mutation in ten-eleven-translocation oncogene family member 2 (*TET2*) has been extensively investigated in the context of several hematologic malignancies and occurs in 7%-23% of patients with acute myeloid leukemia (AML).[1-4] *TET2* acts to demethylate DNA, and *TET2* mutations are leukemogenic in animal models.[5-8] Any prognostic role played by *TET2* mutation in a patient with normal karyotype (NK)-AML remains a subject of debate.[1,2,9-11] Although the prevalence of homozygous *TET2* mutation is 14.8% in patients with *TET2*-mutated myelodysplastic syndrome, and 9.3% in patients with *TET2*-mutated AML, the prognostic role played by this genotype in the context of treatment outcomes has not been investigated in depth.[9,12]

In this study, we evaluated the prevalence of *TET2* mutations in patients with NK-AML and tried to clarify the prognostic role played by *TET2* mutation in patients with NK-AML, especially in those patients with homozygous mutation.

In total, 407 patients were included in the present study, and all met the following eligibility criteria: i) age ≥15 years; ii) a diagnosis of NK-AML confirmed by conventional cytogenetic analysis; and iii) treatment with induction chemotherapy using a standard protocol (a 3-day course of anthracycline with a 7-day course of cytosine arabinoside). Patients with NK-AML were diagnosed between October 1998 and September 2012 in seven participating institutes. Patients who achieved complete remission (CR) received consolidation chemotherapy with or without allogeneic stem cell transplantation (HCT), depending on the availability of a matched related or unrelated donor. Cryopreserved bone marrow (BM) or peripheral blood samples taken at diagnosis were archived before genomic DNA extraction using QIAamp DNA blood mini-kits (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Mutation analysis was performed using Sanger sequencing according to PCR methodology. The sequencing was performed using an ABI 3130xl genetic analyzer (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's protocol. *TET2*, fms-related tyrosine kinase 3-internal tandem duplication (*FLT3*-ITD), and nucleophosmin1 (*NPM1*) mutation testing was performed as previously described.[9,12,13] Homozygosity of *TET2* mutations was considered to be present when the mutant nucleotide signal peak was of a single color and equal in height to adjacent nucleotides. Analysis of the CCAAT/enhancer binding protein α (*CEBPA*) gene is described in *Online Supplementary Table S1*. More details of the CR criteria, survival end point, and statistical analysis are provided in the *Online Supplementary Appendix*.

In total, 407 samples were evaluated in terms of *TET2* mutation. Sixty-five different *TET2* mutations were detected in 54 patients (13.2%) (*Online Supplementary Figure S1*). Single and double *TET2* mutations were detected in 27 patients, and 14 homozygous mutations were observed in patients with double mutations. Details are shown in *Online Supplementary Table S2*.

TET2 mutation was associated with poor prognostic features, such as older age ($P<0.001$) or a high white blood cell (WBC) count ($P=0.013$) (*Online Supplementary Table S3*). *NPM1* mutation was observed more frequently in patients with *TET2* mutations ($P=0.017$). Of 407 patients receiving induction chemotherapy, CR was achieved by 332 (81.6%); no significant difference in CR rates was noted between groups with and without *TET2* mutations (75.9% *vs.* 82.4%, respectively; $P=0.250$). Allogeneic HCT was performed in 32.1% of the whole patient study cohort, but there was no difference in *TET2* mutation frequency between those who did and those who did not receive allogeneic HCT ($P=0.154$). At the median follow-up time of 59.4 months (range: 0.9-179.8 months) among survivors, long-term outcomes were ana-

Table 1. Multivariate analysis of overall survival, event-free survival, and relapse incidence in patients with acute myeloid leukemia with risk factors including homozygous *TET2* mutations.

Parameter	Variable	Hazard ratio	95% CI	*P*
Relapse	Age (> 65 years)	1.411	0.910–2.187	0.120
	Allogeneic HCT	0.372	0.253–0.546	<0.001
	NPM1 mutation	0.477	0.329–0.692	<0.001
	FLT3-ITD mutation	2.249	1.574–3.213	<0.001
	CEBPA mutation	0.536	0.322–0.892	0.016
	Homozygous *TET2* mutation	1.519	1.105–2.086	<0.001
EFS	Age (>65 years)	0.898	0.594–1.357	0.609
	Allogeneic HCT	0.367	0.228–0.593	<0.001
	NPM1 mutation	0.478	0.351–0.653	<0.001
	FLT3-ITD mutation	2.162	1.574–2.970	<0.001
	CEBPA mutation	0.564	0.381–0.835	0.004
	Homozygous *TET2* mutation	1.272	0.862–1.878	0.225
OS	Age (>65 years)	0.963	0.628–1.477	0.864
	Allogeneic HCT	0.382	0.232–0.627	<0.001
	NPM1 mutation	0.518	0.329–0.692	<0.001
	FLT3-ITD mutation	2.216	1.574–3.213	<0.001
	CEBPA mutation	0.621	0.322–0.892	0.020
	Homozygous *TET2* mutation	1.207	0.799–1.825	0.472

OS: overall survival; EFS: event-free survival; TET: ten-eleven translocation; HCT: hematopoietic cell transplantation; NPM1: nucleophosmin; FLT3-ITD: fms-related tyrosine kinase 3-internal tandem duplication; CEBPA: CCAAT/enhancer binding protein.

lyzed by *TET2* mutational status. In all patients (n=407), no difference in relapse incidence (RI; 47.6% *vs.* 43.3% at 5 years; *P*=0.717), event-free survival (EFS; 28.0% *vs.* 34.5% at 5 years; *P*=0.391), or overall survival (OS; 35.8% *vs.* 37.4% at 5 years; *P*=0.581) was noted between patients with or without *TET2* mutations (*Online Supplementary Table S4* and *Online Supplementary Figure S2* and). Upon multivariate analysis of factors affecting RI, EFS, and OS, independent risk factors for RI, EFS, and OS were performance of allogeneic HCT and mutations in *NPM1, CEBPA*, or *FLT3*-ITD, but neither a *TET2* mutation alone nor older age had any prognostic impact on RI, EFS, or OS (*Online Supplementary Table S5*).

Homozygous *TET2* mutations were detected in 14 (25.9%) patients with *TET2* mutations. The *NPM1* mutation was observed more frequently in patients with non-homozygous *TET2* mutations than in patients with homozygous *TET2* mutations (*P*=0.017). However, there was no difference in the clinical features and other molecular mutational status between patients with homozygous and non-homozygous *TET2* mutations (*Online Supplementary Table S6*). We categorized the *TET2* mutational status as wild-type, single mutation, and heterozygous double or homozygous mutation; allogeneic HCT was performed in 33.7%, 25.9%, 23.1% and 14.2% of each group without any statistical difference being found (*P*=0.378). In OS and EFS, there was no statistical difference among *TET2* wild-type, *TET2* single mutation, and heterozygous double *TET2* mutation. However, patients with homozygous *TET2* mutation showed significantly inferior EFS compared with those with wild-type *TET2* (*P*=0.048). Importantly, patients with homozygous *TET2* mutation showed a higher relapse rate compared with those with wild-type *TET2* (RI at 5 years: 100.0% *vs.* 43.1%; *P*=0.002) or single *TET2* mutations (RI at 5 years: 100.0% *vs.* 41.1%; *P*=0.012) or *TET2* heterozygous double mutation (RI at 5 years: 100.0% *vs.* 27.3%; *P*=0.023). However, the patients with single or heterozygous double *TET2* mutations showed similar relapse rates at five years compared with those with wild-type *TET2* (36.4% *vs.* 42.4%; *P*=0.673) (*Online Supplementary Table S7*). Homozygous *TET2* mutation was an independent adverse prognostic factor for RI in multivariate analysis (HR: 1.519: 95%CI: 1.105-2.089; *P*<0.001) (Table 1); however, such status appeared not to affect EFS and OS.

Notably, we found that the RI of patients with NK-AML with homozygous *TET2* mutations was significantly higher than that of patients with non-homozygous *TET2* mutation or wild-type *TET2*. In animal models, *TET2*-haploinsufficient mice were similar to mice with homozygous *TET2* null mutations, in that both types of animal developed various hematopoietic malignancies.[5,6,8] However, also in mouse models, BM cells with heterozygous *TET2* null mutations exhibited *TET2*-encoding mRNA expression levels of 40%-50% those of wild-type animals, whereas no such mRNA was synthesized in homozygous *TET2*-null mice.[8] The 5-hydroxymethylcytosine level reflects dynamic epigenetic changes in DNA, and was reduced dramatically in homozygous *TET2*-null mice compared to heterozygous *TET2*-null and wild-type animals. Approximately 33% of homozygous *TET2*-null and 8% of heterozygous *TET2*-null mice developed lethal myeloid malignancies in the first year of life, suggesting that disease latency was much longer in heterozygous *TET2*-null mice than in homozygous *TET2*-null animals.[8] Such results imply that *TET2* loss triggers dose-dependent effects on hematopoiesis and myeloid transformation. In other studies, at least one *TET2* mutant allele was present in most cells (>70%), and biallelic mutation was also observed frequently in 26%-42%.[12,14,15] However, the mutant allele burden or monoallelic *versus* biallelic *TET2* mutation did not significantly correlate with poorer clinical prognosis or OS.[9,12,15] We recorded homozygous *TET2* mutations in 25.9% of NK-AML patients with *TET2* mutations, and this was strongly correlated with higher RI even in comparison with the non-homozygous *TET2* mutation group, suggesting that the homozygous *TET2* mutation may be associated with a short interval prior to leukemic relapse, thus exhibiting a "threshold effect."

Our present study had several limitations as regards the interpretation of the clinical significance of homozygous *TET2* mutation. First, our study had a methodological limitation in that the detection sensitivity of Sanger fluorescent dideoxynucleotide chain termination sequencing analysis is approximately 10% of mutant alleles. Using sequencing analysis, it is difficult to determine and distinguish biallelic mutations from double mutations because of this relatively low sensitivity, depending on the sequencing trace source. Second, the work was retrospective in nature, included patients treated in several centers, and the consolidative therapies received by patients were not homogeneous. Third, the sample size

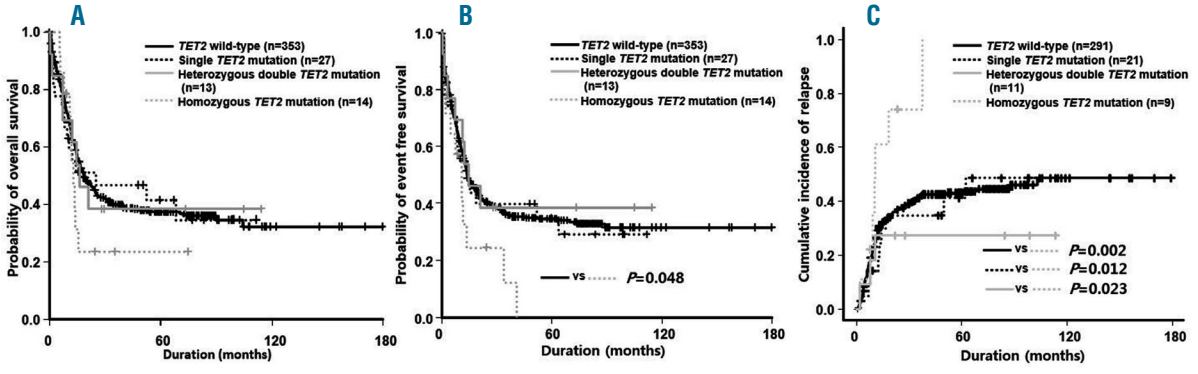

Figure 1. The prognostic significance according to *TET2* mutational status. Overall survival (A), event-free survival (B), and estimated relapse incidence (C).

of the group with homozygous *TET2* mutations was small, limiting the power of direct comparisons with patients bearing non-homozygous *TET2* mutations or *TET2* wild-type. However, our work has significant clinical relevance in that we included a large number of patients with NK-AML only. Our work clarifies the prognostic significance of the *TET2* mutation in treated populations, and helps clarify the prognostic significance of such mutation in patients with NK-AML. In addition, the present study is the first to investigate the significance of homozygous *TET2* mutational status in patients with NK-AML.

In conclusion, such non-homozygous *TET2* mutations did not influence treatment outcomes. However, homozygous *TET2* mutational status was prognostic in terms of a higher RI, suggesting that the *TET2* mutation exerts a "threshold effect" in the context of relapse. Further study of larger numbers of patients will yield valuable data on the prognostic role played by the *TET2* mutation in terms of treatment outcomes in NK-AML. Additional biological validation work is also required to explore the prognostic role played by homozygous *TET2* mutational status in terms of risk of leukemia relapse.

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